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Genetic Analysis of Colistin Resistance in *Salmonella enterica* Serovar Typhimurium[▽]†

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Colistin is a cyclic cationic peptide that kills gram-negative bacteria by interacting with and disrupting the outer membrane. We isolated 44 independent mutants in *Salmonella enterica* serovar Typhimurium with reduced susceptibility to colistin and identified 27 different missense mutations located in the *pmrA* and *pmrB* genes (encoding the regulator and sensor of a two-component regulatory system) that conferred increased resistance. By comparison of the two homologous sensor kinases, PmrB and EnvZ, the 22 missense mutations identified in *pmrB* were shown to be located in four different structural domains of the protein. All five *pmrA* mutations were located in the phosphate receiver domain of the regulator protein. The mutants appeared at a mutation rate of 0.6×10^{-6} per cell per generation. The MICs of colistin for the mutants increased 2- to 35-fold, and the extent of killing was reduced several orders of magnitude compared to the susceptible strain. The growth rates of the mutants were slightly reduced in both rich medium and M9-glycerol minimal medium, whereas growth in mice appeared unaffected by the *pmrA* and *pmrB* mutations. The low fitness costs and the high mutation rate suggest that mutants with reduced susceptibility to colistin could emerge in clinical settings.

Polymyxins (PMs) constitute a group of membrane-active polypeptide antibiotics that is comprised of five chemically different compounds (PMs A to E) (34). Colistin (PM E) was discovered in 1949 and was later cautiously used clinically because of the reported high incidence of nephrotoxicity (14). However, the emergence of multidrug-resistant gram-negative bacteria and the serious lack of development of new antibiotics led to a revival in the use of PMs, and several recently published studies and case reports have shown that colistin can be used safely and effectively with minimal adverse outcomes (12, 25, 29). Two forms of colistin are available commercially: colistin sulfate and sodium colistin methanesulfonate. The predominant use of these drugs over the last 20 years has been for inhalational treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis patients (10). Recently, there have been several clinical reports on the efficacy of intravenous colistin methanesulfonate in patients with infections by different types of gram-negative bacteria (17, 19). Most attention has been focused on multiresistant strains of *P. aeruginosa* and *Acinetobacter baumannii*, but colistin is also used for the treatment of *Klebsiella pneumoniae* and *Enterobacter cloacae* infections (11, 35).

Colistin targets the cell envelope, and the interaction between the cationic polypeptide and negatively charged lipopolysaccharides (LPS) leads to a disturbance of the outer membrane and a subsequent increase in the permeability of the cell envelope that ultimately results in death (21, 30). In vitro studies of PM-resistant mutants of *Salmonella enterica* serovar Typhimurium have shown that the resistance is primarily caused by decreased binding of PM to the mutant LPS (37). Mutations conferring colistin resistance on *S.* Typhimurium were mapped to a locus named *pmrA* (18). In *S.* Typhimurium, *P. aeruginosa*, and *A. baumannii*, two genes, *pmrA* and *pmrB*, were identified and shown to constitute a two-component regulatory system that is responsible for PM resistance in all three species (1, 20, 28). This similarity implies that studies of the genetically amenable species *S.* Typhimurium could be applicable to the clinically relevant species *P. aeruginosa* and *A. baumannii*.

PmrB is a sensor histidine kinase, and PmrA is the cognate response regulator. The activity of the PmrA-PmrB two-component system can be modulated by either environmental signals or mutational change in the *pmrA* and *pmrB* genes (20, 28, 33, 38). After being phosphorylated by PmrB, PmrA can effectively bind to the transcriptional sites of other genes (*pmrE*, *pmrHFIJKLM*, and *pmrC*) and activate their transcription. As shown for both *S.* Typhimurium and *P. aeruginosa*, the proteins encoded by these genes modify the LPS core and lipid A regions with ethanolamine and add aminoarabinose to lipid A (7, 8, 16, 20). These modifications make the LPS less negatively charged and thereby decrease the binding of colistin. This work is focused on the mutational study of the development of colistin resistance. To our knowledge, until now only one missense mutation (R81H) in the *pmrA* gene in *S.* Typhimurium and two missense mutations (L243Q and A248V) in the *pmrB* gene in *P. aeruginosa* have been identified (20, 28). Although it was suggested that PM resistance could result from mutations in the *pmrB* gene that lead to a hyperactive kinase or decreased phosphatase activity, few mutations have been identified, and how these mutations affect the PmrA-PmrB two-component regulatory system is still unclear (20, 28).

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Antibiotic resistance typically causes perturbations in the activities of cellular processes that often have deleterious effects on cellular fitness, quantifiable as changes in fitness parameters, such as virulence, growth rates, and transmission rates (2). Both experimental and theoretical data suggest that the biological fitness cost of resistance is a major determinant of the rate of resistance development, and knowledge of the fitness costs is thus important if we want to be able to predict resistance development in different clinical settings, as well as to evaluate the effects of interventions to reduce antibiotic resistance.

We isolated 44 spontaneous mutants of *S.* Typhimurium LT2 that showed reduced susceptibility to colistin sulfate (referred to as colistin throughout the text) and carried mutations in either the *pmrA* or *pmrB* gene. We determined the mutation rate to colistin resistance and measured the fitness of the *pmrAB* mutants in vitro and in a mouse model.

MATERIALS AND METHODS

Isolation of colistin-resistant mutants. For all experiments, *S.* Typhimurium LT2 (referred to as *S.* Typhimurium) was used. One motivation for using *S.* Typhimurium in this study (apart from the fact that it is a genetically amenable experimental system with a robust and widely used animal infection model) is the fact that many of the previous studies of the PmrAB system have been performed in this bacterium (8, 18, 28), allowing us to make proper comparisons between our study and previous studies. From each of several independent overnight cultures, 2×10^7 cells were plated on Luria agar (LA) plates supplemented with 1.25 mg/liter (10 times the MIC of colistin for the susceptible strain) or 6.25 mg/liter (50 times the MIC of colistin for the susceptible strain) colistin. Forty-four independent mutants with reduced susceptibility to colistin, 27 from plates containing 6.25 mg/liter colistin and 17 from plates containing 1.25 mg/liter colistin, were isolated, restreaked on LA-colistin plates, and then stored at −80°C.

Determination of the in vitro mutation rate. Approximately 10^8 CFU from each of 25 independent cultures were inoculated onto LA plates containing 1.25 mg/liter colistin, and the number of colonies with reduced susceptibility to colistin was scored the next day. The mutation rate was calculated as the median number of colonies with reduced susceptibility divided by the total number of plated cells as described previously (15).

Determination of MIC and cross-resistance. The MICs of colistin for the mutants were determined using Etest strips from AB Biodisk as described by the manufacturer. Each MIC was determined in at least three separate experiments. Cross-resistance to five antimicrobial peptides (AMPs), CNY100, LL-37, PR-39, protamine, and bleomycin, was determined in 96-microwell plates (round bottom; Nunc A/S, Roskilde, Denmark). The susceptible wild type (DA6192), two *pmrA* mutants (DA10826 and DA10840), and two *pmrB* mutants (DA10833 and DA10845) were grown to mid-exponential phase and diluted to approximately 10^5 CFU/ml, and then 90 μl of the diluted bacteria was mixed with various amounts of AMPs to a final volume of 100 μl. The plates were incubated at 37°C with shaking at 200 rpm overnight. The MIC was defined as the lowest concentration of AMPs giving no visible growth.

Time-kill assay. Two *pmrA* (DA10826 and D10840) and two *pmrB* (DA10829 and DA10845) spontaneous mutants and the wild-type strain (DA6192) were used to examine the killing kinetics of colistin. About 10^6 cells in mid-exponential phase (optical density [OD] = 0.5) were inoculated into 10 ml of LB broth in a flask supplemented with 0, 0.25, 1, 4, or 16 mg/liter colistin. The flasks were shaken at 200 rpm at 37°C. Samples were taken at five time points, zero (prior to the addition of antibiotic), 10 min, 30 min, 240 min, and 480 min, and viable counts were determined for each time point.

PCR and sequencing. The primers for the relevant genes were designed using NetPrimer. For DNA sequencing, the gene of interest was PCR amplified and purified from solution using GFX PCR DNA and a gel band purification kit (GE Healthcare). The purified PCR product was mixed with the sequencing primers, and the samples were dried in a vacuum and sent to the MWG Biotech Company, Germany, for DNA sequencing. The sequences were analyzed using Mega 3.1. The primers used for amplification and sequencing were as follows: *S.* Typhimurium LT2 *pmrA*, Fwd (5′-CGC GAA TTT CGT GCA TGA TAT G-3′) and Rev (5′-ATG TCC CGA TGC TCA TTT GGC-3′), and *pmrB*, Fwd (5′AGG AAA TTC TGG GCG AGC A-3′) and Rev (5′-CGT TTT CAG CGA AGA GCG A-3′).

Measurements of growth rates and starvation survival. Exponential growth rates were measured in LB broth and M9-glycerol (0.2%) minimum medium at 37°C. In a Bioscreen plate, approximately 10^4 and 10^8 cells were inoculated into 400 μl LB medium and M9-glycerol medium, respectively, and the absorbance at 600 nm was read every 4 min using a BioscreenC reader. Growth rates were calculated based on OD values at 600 nm measured between 0.08 and 0.15 in the exponential phase. Relative growth rates were calculated as the growth rates of the mutants divided by the growth rate of the susceptible wild-type strain (DA6192) from the same experiment. Standard errors were ±2% of the average, and differences greater than 2% were considered significant. The starvation survival of the mutants was determined by incubating four independent lineages (each) of the strains DA6192 (wild type), DA10826, DA10840, DA10833, and DA10853 in LB over an extended period without changing the medium. Each independent lineage was initiated by inoculating 1.5 ml LB with 10^5 bacterial cells. The tubes were incubated at 37°C for 21 days, and samples were plated on LA plates at regular intervals. The survival percentage compared to the number of CFU after an overnight incubation was calculated for each sampling time point. Standard errors were ±50% of the average values, and the small differences in survival (see Fig. 3) were not significant.

Animal experiments. The in vivo fitness of two *pmrA* (DA10826 and D10840) and two *pmrB* (DA10833 and DA10853) mutants was measured by performing competition experiments in mice. These experiments were carried out at the animal facility at the Microbiology and Tumor Biology Centre, Karolinska Institute (Stockholm, Sweden), in compliance with national and institutional guidelines (ethical permit N107/06). Each bacterial strain was grown to logarithmic growth phase, diluted, and mixed with strain DA6080 (a genetically tagged reference competitor that carries a selectively neutral MudJ element with a kanamycin resistance gene inserted in the *cob* operon [22]) at an approximately 1:1 ratio in phosphate-buffered saline (PBS). For each competition experiment, five 7-week-old female BALB/c mice were infected with ~2×10^5 cells in a 100-μl volume of PBS by intraperitoneal injection. The mice were sacrificed after 3 days, and the livers and spleens were removed and homogenized in PBS. The ratio of mutants versus the wild-type reference strain was determined by plating different dilutions of the homogenates on LA plates and LA plates supplemented with kanamycin. The competition index (CI) was calculated as the ratio between the mutant and wild-type populations after one cycle of growth in mice (~10 generations). From this value and the number of generations of growth in mice, the selection coefficient (s) was calculated as follows: $s = \ln(\mathrm{CI})/10 \ln(2)$ (22). Because of variation between mice, only differences in the CI greater than two- to threefold were considered significant.

Isolation of total RNA. Total RNA was isolated using the SV Total RNA Isolation System (part no. TM048) from Promega. Bacterial cell lysis was done as follows. A culture was grown overnight in LB broth at 37°C, and the following day, the culture was diluted 1:100 and grown at 37°C until the OD values at 600 nm were around 0.4 to 0.5. A pellet was collected by centrifuging 1.4 ml culture at 13,200 rpm and was then resuspended in 100 μl of freshly prepared Tris-EDTA containing 0.5 g/liter lysozyme. The subsequent steps were as described in the user manual.

First-strand synthesis of cDNA from mRNA. mRNA was converted to cDNA using the cDNA reverse transcription kit from Applied Biosystems. Reverse transcription-PCRs were performed by mixing 10 μl isolated total RNA with 10 μl 2× reverse transcription master mix. The protocol for preparing 2× reverse transcription master mix and the thermal-cycling program were according to the manufacturer's suggestions.

Quantitative real-time PCR. The quantitative real-time PCR technique based on the high affinity of SYBR Green dye for double-stranded DNA was used. The fluorescence signal was monitored on line using a MiniOpticon real-time PCR system (Bio-Rad Laboratories). The PCR amplification was performed by mixing 12.5 μl cDNA and primers with 12.5 μl SYBR Green SuperMix (Bio-Rad Laboratories). The primers used for real-time PCR were as follows: *S.* Typhimurium LT2 *pmrH* real time, Fwd (5′-TGT CGG CTA ACT GGC AAT CA-3′) and Rev (5′-GTA ATG ACC TCG TCG CCT TC-3′), and *recA* real time, Fwd (5′-TAC CGA ACA TCA CGC CAA TC-3′) and Rev (5′-GTA TGA TGA GCC AGG CGA TG-3′).

Nucleotide sequence accession numbers. The DNA sequences of the *pmrA* (accession no. NC_003197) and *pmrB* (accession no. NC_003197) genes have been deposited in GenBank.

TABLE 1. Genotypes and MICs of colistin for strains used in this study

Strain	Genotype[a]	MIC (mg/liter)	Origin
DA6192	Wild type (parent)	0.125	Strain collection
DA10844	*pmrA* (ggg→agg, G15R)	2.7	This study
DA10826	*pmrA* (GGG→GAG, G53E)	3	This study
DA10850	*pmrA* (GGG→AGG, G53R)	3	This study
DA10857	*pmrA* (CGC→TGC, R81C)	4	This study
DA10840	*pmrA* (CGC→CAC, R81H)	3	This study
DA10865	*pmrB* (TTA→TCA, L14S)	3.5	This study
DA10843	*pmrB* (TTTA→TTC, L14F)	3.5	This study
DA10849	*pmrB* (CTG→CCG, L22P)	3.5	This study
DA10829	*pmrB* (AGT→AGA, S29R)	0.5	This study
DA10832	*pmrB* (ACC→GCC, T92A)	0.25	This study
DA10837	*pmrB* (CCG→CAG, P94Q)	2.5	This study
DA10862	*pmrB* (GAG→GCG, E121A)	4	This study
DA10839	*pmrB* (TCC→CCC, S124P)	2.3	This study
DA10853	*pmrB* (AAT→TAT, N130Y)	2	This study
DA10874	*pmrB* (ACC→CCC, T147P)	2.5	This study
DA10838	*pmrB* (CGC→CCC, R155P)	3.5	This study
DA10848	*pmrB* (ACG→CCG, T156P)	4.4	This study
DA10854	*pmrB* (ACG→ATG, T156 M)	3.5	This study
DA10856	*pmrB* (GTG→ATG, V161 M)	4	This study
DA10835	*pmrB* (GTG→CTG, V161L)	2	This study
DA10859	*pmrB* (GTG→GGG, V161G)	3	This study
DA10833	*pmrB* (GAA→AAA, E166K)	3	This study
DA10845	*pmrB* (ATG→ATA, M186I)	2.8	This study
DA10863	*pmrB* (GGG→TGG, G206W)	2	This study
DA10875	*pmrB* (GGG→AGG, G206R)	2	This study
DA10831	*pmrB* (AGC→CGC, S305R)	2.7	This study
DA10864	*pmrB* (AGC→AGA, S305R)	2.7	This study

[a] DNA changes and amino acid substitutions (using the one-letter code) in the PmrA and PmrB proteins are indicated.

RESULTS AND DISCUSSION

Isolation and characterization of mutants with reduced susceptibility to colistin. In this work, we isolated 44 spontaneous mutants with reduced susceptibility to colistin. The mutation rate to colistin resistance was calculated to be 0.6×10^{-6} per cell per generation by using the median method of Lea and Coulson (15), which is several orders of magnitude higher than the mutation rates to resistance for other antibiotics, such as streptomycin, rifampin (rifampicin), and nalidixic acid (27).

The MICs of colistin were determined for the spontaneous mutants and the wild-type strain, and for most mutants, the MIC was increased about 20- to 30-fold compared to the susceptible strain (Table 1). The susceptible strain and four mutants with slightly different MICs of colistin were selected to investigate the bactericidal effect of colistin. The CFU for each culture was determined as a function of time (Fig. 1). For the susceptible strain, a bactericidal effect was observed at the 1-mg/liter colistin concentration after 30 min; for the three less susceptible mutants, except DA10826, a bactericidal effect was observed after 30 or 240 min at a 16-mg/liter colistin concentration; for the mutant DA10826, there was only a fivefold reduction in viability after 480 min at 16 mg/liter colistin. The mutants with high MICs of colistin were more resistant to its killing effect.

The cross-resistances of two *pmrA* mutants (DA10826 and DA10840) and two *pmrB* mutants (DA10833 and DA10845) to five other AMPs were determined. No cross-resistance to either CNY100 H-L, PR-39, protamine, or bleomycin was observed, but the MICs of LL-37 increased slightly but reproducibly for all four mutants (MIC = 25 μM) compared to the wild-type strain (MIC = 15 μM) (Table 2).

Identification of colistin resistance mutations. DNA sequencing of the *pmrA* (region 4533719 to 4534387) and *pmrB* (region 4532639 to 4533709) genes from 44 spontaneous mutants showed that 36 mutants had mutations in the *pmrB* gene, representing a total of 22 different base pair substitutions that resulted in 22 amino acid changes located at 16 different positions, and 8 mutants had base pair changes in the *pmrA* gene that resulted in 5 different amino acid substitutions located at 3 different positions (Table 1). To confirm that these mutations were required and sufficient to confer colistin resistance, they were transferred by phage P22 transduction using a linked kanamycin resistance marker (located in the *adi* gene) to a wild-type genetic background. After transfer, a fraction (representing the linkage between the kanamycin resistance marker and the *pmrAB* genes) of the transductants became less susceptible to colistin, confirming that the *pmrAB* mutations alone conferred the resistance (data not shown).

Among the 44 mutants examined, we found 27 different types of mutations, indicating that we had identified a substantial fraction of all the mutations that can confer the resistance phenotype. From the multiple-recovery frequency of mutations, the total number of possible mutations can be calculated. If all mutations occur at the same rate, the distribution of the number of occurrences is expected to be Poissonian. Thus, if 27 different mutations have been observed among 44 independently selected mutations, the number of possible resistance mutations (m) can be estimated from the equation $27 = m(1 - e^{-44/m})$ to be $m = 41$ if one assumes that all mutations occur at the same rate and that their fitness effects are similar. As shown in Fig. 2, this assumption is reasonable, and the fitness differences observed will have a negligible effect on mutant recovery in a Luria-Delbruck fluctuation test.

Fitness of mutants with reduced susceptibility during growth in vitro. The growth rates of 5 *pmrA* mutants and 18 *pmrB* mutants were measured in LB medium and M9-glycerol growth medium without colistin (Fig. 2). In LB medium, the relative growth rates of the *pmrB* mutants (compared to that of the wild type, which was set to 1.0) varied from 0.93 to 1.02, and the average fitness was 0.98, indicating a 2% average reduction compared with the wild type (unpaired t test; $P = 0.018$). The relative growth rates of the *pmrA* mutants varied from 0.92 to 0.96, and the average fitness was 0.94, indicating a 6% reduction compared with the wild type (unpaired t test; $P = 0.00013$). Compared with the *pmrB* mutants, the average fitness of the *pmrA* mutants was reduced by 4% (unpaired t test; $P = 0.0001$). In M9-glycerol minimum medium, a similar but more pronounced pattern was observed. Thus, the relative growth rates of the *pmrB* mutants varied from 0.78 to 1.1 and the average fitness was 0.87, a 13% reduction compared to the wild type (unpaired t test; $P = 0.0004$). Furthermore, the relative growth rates of the *pmrA* mutants varied from 0.78 to 0.89 and the average fitness was 0.82, a 18% reduction compared to the wild type (unpaired t test; $P = 0.00001$). In M9-glycerol medium, the average fitness of the *pmrA* mutants was 6% lower than that for the *pmrB* mutants (unpaired t test; $P = 0.07$). The fitness costs of reduced susceptibility to colistin were more significant in poor medium than in rich medium, and in

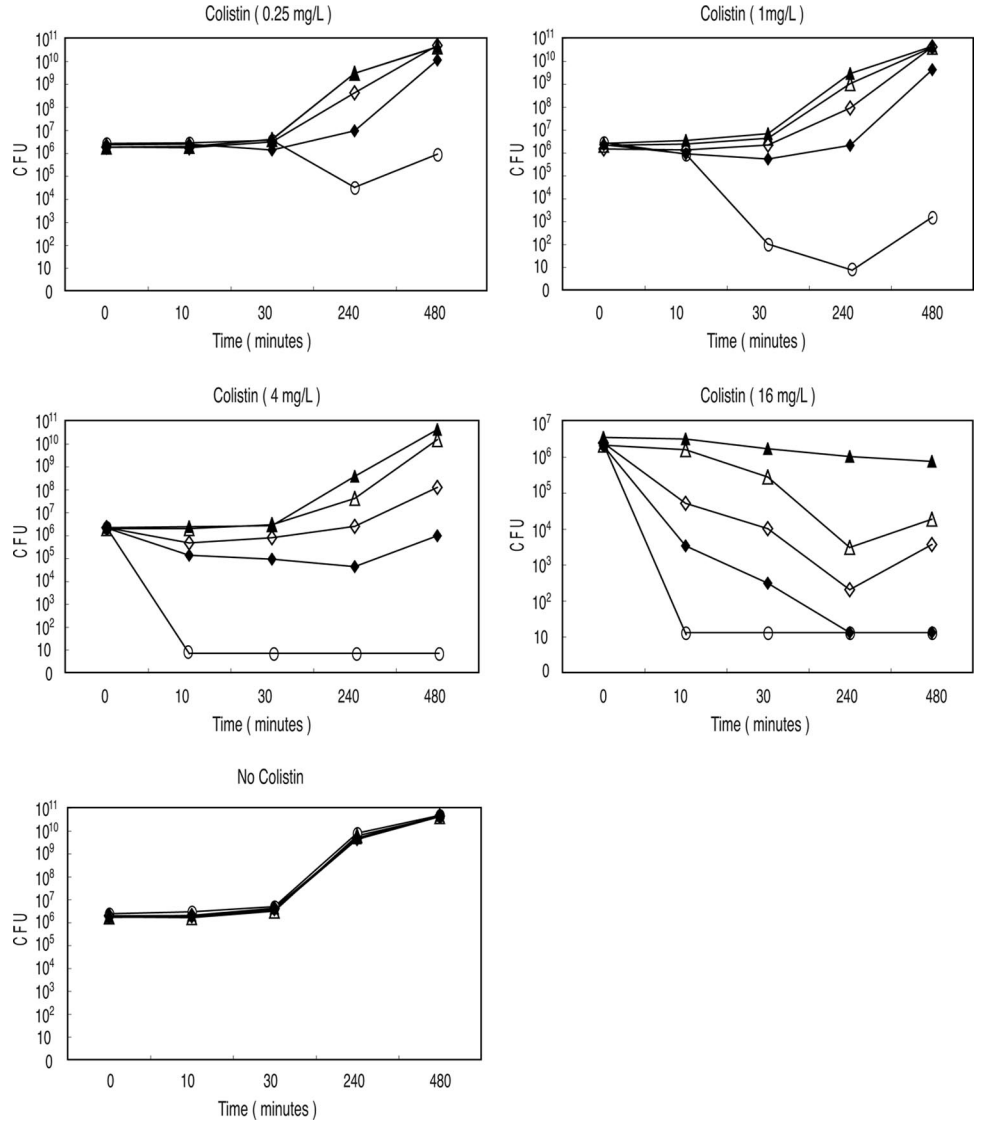

FIG. 1. Killing curves for the wild type and four mutants with reduced susceptibility to colistin. CFU are plotted as a function of time for different concentrations of colistin (0.25, 1, 4, and 16 mg/liter). Amino acid substitutions in the mutants (using the one-letter code) are indicated as follows: open circles, DA6192 (wild type); filled diamonds, DA10829 (PmrB [S29R]); open diamonds, DA10845 (PmrB [M186I]); filled triangles, DA10826 (PmrA [G53E]); open triangles, DA10840 (PmrA [R81H]).

both rich and minimal media, the *pmrA* mutants showed a more severe reduction in growth than the *pmrB* mutants.

Since the growth rate in exponential phase is only one parameter that determines overall fitness, we also assessed the potential of these mutants to survive prolonged starvation. Four independent lineages of DA6192 (wild type), DA10826 (*pmrA*), DA10840 (*pmrA*), DA10833 (*pmrB*), and DA10853 (*pmrB*) were inoculated into LB broth. The numbers of viable

TABLE 2. Susceptibilities of mutants with reduced susceptibility to colistin to different AMPs

Peptide	MIC determined in liquid culture[a]				
	DA6192 (wild type)	DA10826 (PmrA [G53E])	DA10833 (PmrB [E166K])	DA10840 (PmrA[R81H])	DA10845 (PmrB [M186I])
CNY 100 H-L	12.5 μM	12.5 μM	12.5 μM	12.5 μM	12.5 μM
PR-39	3 μM	3 μM	3 μM	3 μM	3 μM
Protamine sulfate	31 mg/liter	31 mg/liter	31 mg/liter	31 mg/liter	31 mg/liter
Bleomycin	0.19 mg/liter	0.19 mg/liter	0.19 mg/liter	0.19 mg/liter	0.19 mg/liter
LL-37	15 μM	25 μM	25 μM	25 μM	25 μM

[a] Amino acid substitutions in the mutants (using the one-letter code) are indicated.

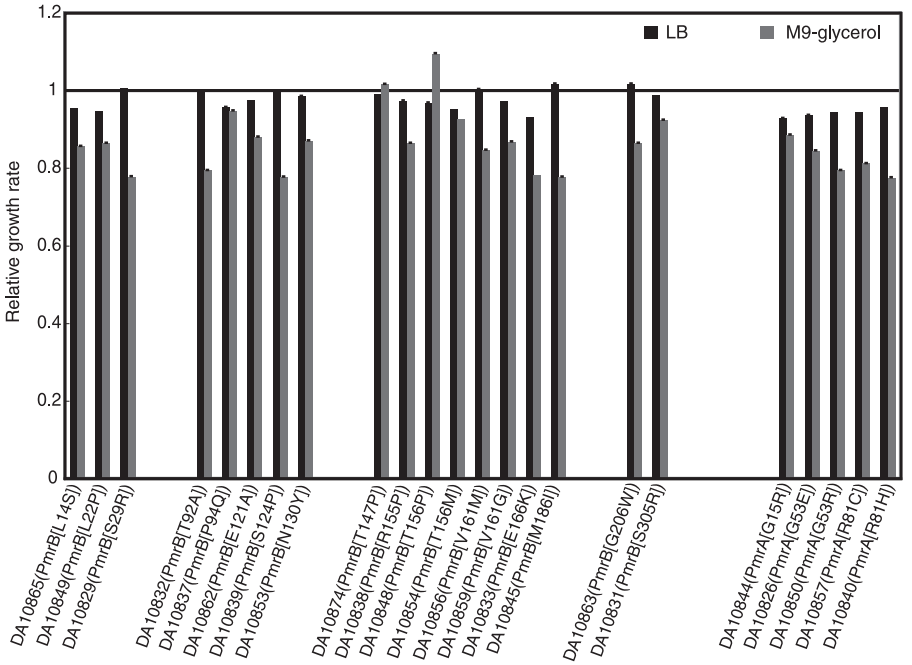

FIG. 2. Relative growth rates of mutants with reduced susceptibility to colistin measured in LB broth and M9-glycerol medium. The growth rate for each strain was determined from four to eight independent cultures. The standard deviation of the relative growth rates is ±2%.

bacteria at different time points were determined by plating samples on LA plates, and the survival percentage was calculated as the number of survivors divided by the initial number of colonies in each culture (Fig. 3). No significant differences were observed between the four mutants and the wild-type strain. The number of viable cells decreased to about 0.2 to 0.7% of the original population in 21 days.

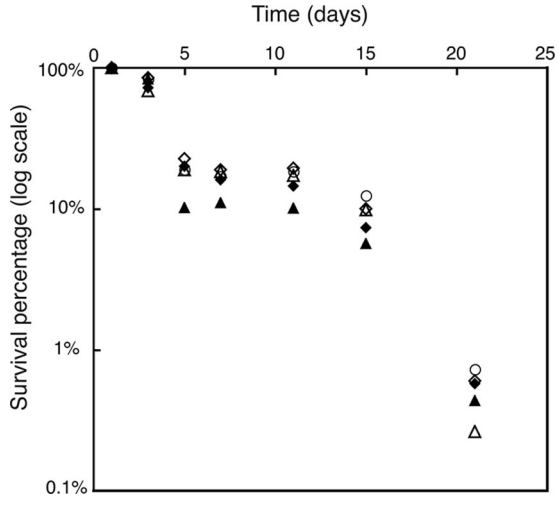

FIG. 3. Stationary-phase survival of four strains with reduced susceptibility to colistin compared to that of the fully susceptible strain. Four independent cultures of DA6192 (wild type), DA10826 (PmrA [G53E]), DA10840 (PmrA [R81H]), DA10833 (PmrB [E166K]), and DA10853 (PmrB [N130Y]) were incubated at 37°C for 21 days. The survival percentage was calculated as the average number of viable cells at each time point divided by the number of viable cells at the start of the experiment. Amino acid substitutions in the mutants (using the one-letter code) are indicated as follows: open circles, DA6192 (wild type); filled triangles, DA10826 (PmrA [G53E]); open triangles, DA10840 (PmrA [R81H]); open diamonds, DA10833 (PmrB [E166K]); filled diamonds, DA10853 (PmrB [N130Y]).

Fitness of resistant mutants during growth in mice. To determine the effects of the *pmrA* and *pmrB* mutations on fitness in mice, we utilized an in vivo competition assay. This animal model has been used in a number of studies and is regarded as a useful model to assess bacterial fitness in a host (4, 5, 24). Approximately equal numbers of mutant and wild-type bacteria were mixed and inoculated intraperitoneally into five mice. After 3 days, bacteria were harvested from the spleens and livers of the mice and plated on LA and LA-kanamycin plates to determine the number of mutant versus wild-type bacteria. None of the strains DA10826 (*pmrA*), DA10840 (*pmrA*), DA10833 (*pmrB*), and DA10853 (*pmrB*) showed any significant differences in fitness compared to the wild type (Table 3). The relatively low (in vitro) or absent

TABLE 3. CIs and relative growth rates of strains with reduced susceptibility to colistin in mice[a]

Strains	CI		Relative growth rate	
	Liver	Spleen	Liver	Spleen
DA10826 (PmrA [G53E])	0.60	0.55	0.93	0.91
DA10840 (PmrA [R81H])	1.55	1.90	1.06	1.09
DA10833 (PmrB [E166K])	1.21	1.00	1.03	1.00
DA10853 (PmrB [N130Y])	0.63	0.52	0.93	0.91

[a] The CI was calculated as the number of mutant colonies divided by the number of wild-type colonies after 10 generations of growth in mice. The growth rate of the wild-type strain was set to 1, and the relative growth rates of the mutants were calculated as $1 + s$ (the selection coefficient), where $s = \ln(\text{CI})/10 \ln(2)$. Amino acid substitutions in the mutants (using the one-letter code) are indicated.

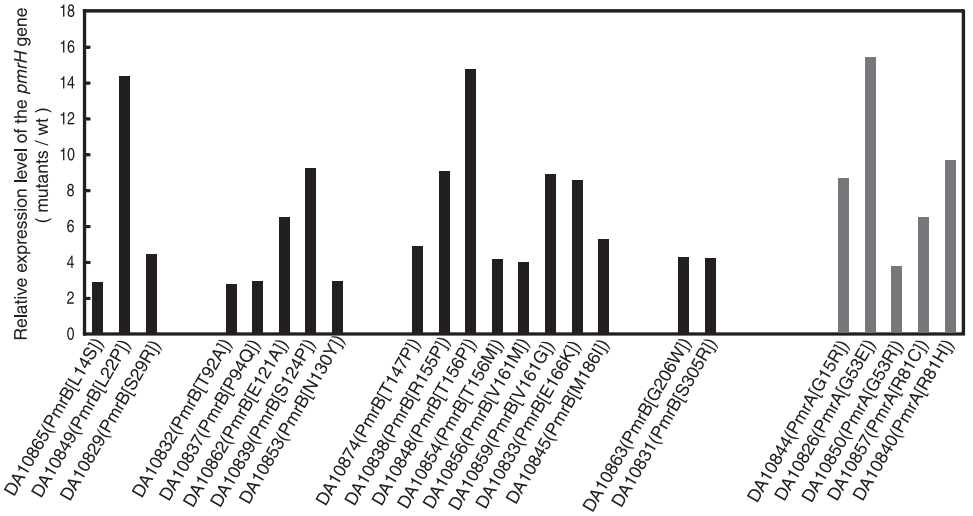

FIG. 4. Relative expression levels of the *pmrH* gene in mutants with reduced susceptibility to colistin expressed as mutant/wild-type (wt) levels. Mutations in the *pmrA* (gray) and *pmrB* (black) genes are indicated. Amino acid substitutions in the mutants (using the one-letter code) are indicated.

(animal model) fitness cost of the *pmrAB* mutations and the high mutation rate suggest that mutants with reduced susceptibility to colistin may be selected for in clinical settings, which also is compatible with the current clinical situation (3, 9, 13, 26, 31, 36).

Expression level of the *pmrH* gene. The expression of the *pmrH* gene has been shown to be regulated by the PmrA-PmrB two-component regulatory system, and the gene is also necessary for conferring PM resistance (7). We measured the expression levels of the *pmrH* gene for 18 *pmrB* mutants and 5 *pmrA* mutants using real-time PCR (Fig. 4). Compared with the wild type, the expression levels of the *pmrH* gene in the mutants with reduced susceptibility were increased 2- to 15-fold, showing that the *pmrA* and *pmrB* mutations changed the regulation of the PmrA-PmrB-dependent genes. There was no clear correlation between the expression level of the *pmrH* gene and the MICs for colistin of the set of mutants examined. Since the PmrH enzyme is only one of several enzymes required for the modification of LPS and the *pmrAB* mutations might affect the expression levels of these other proteins as well, one would not necessarily expect a direct correlation between *pmrH* expression and the extent of LPS modification (8). In addition, the small differences between the MICs of colistin for the different mutants could also make it difficult to reveal a potential correlation.

Identification of the domain locations of the mutations based on homologous protein structure. PmrA and PmrB show homology with other members of two-component regulatory

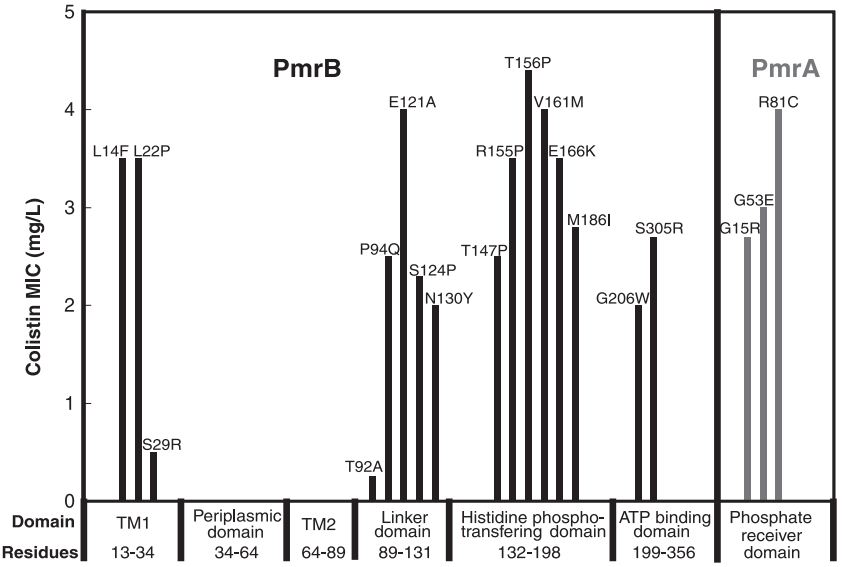

FIG. 5. Sequence locations of all mutations conferring colistin resistance and the MICs of colistin for the corresponding mutants. Mutations in *pmrA* (gray) and *pmrB* (black) and amino acid substitutions in the mutants (using the one-letter code) are indicated.

systems, e.g., EnvZ-OmpR and PhoR-PhoB. By combining the amino acid sequence alignment result (between PrmB and nine other homologous histidine kinases [see Fig. S1 in the supplemental material]) and the well-studied domains of EnvZ (6), PmrB was dissected into six domains: TM1 (transmembrane domain 1), a periplasmic domain, TM2, a linker (HAMP) domain, a histidine-phosphotransfering (HK) domain, and an ATP-binding domain (Fig. 5) (6, 23). The residue numbers for each domain are shown in Fig. 5. The 16 mutated residues in the *pmrB* mutants were distributed over all of the domains except the periplasmic domain and TM2. In several mutational studies of EnvZ, mutations in the TM1 domain, the HAMP domain, and the HK domain have been shown to increase the ratio of kinase activity to phosphatase activity. In this work, we were able to find amino acid changes in the PmrB proteins in these three domains, as well as two mutations in the ATP-binding domain. Thus, it is possible (even though not experimentally demonstrated in this study) that the mutations in the PmrB protein also alter the regulation of the PmrA-PmrB regulatory system by increasing the kinase/phosphatase activity ratio. Based on the alignment data between PmrA and nine other homologous response regulators (see Fig. S1 in the supplemental material) and a structural study of the PhoB protein (32), PmrA was dissected into two domains: the phosphate receiver domain and the DNA binding domain. All *pmrA* mutations were located in the phosphate receiver domain, in the vicinity of the Asp51 active site (32). Thus, one possible rationalization for the effects of these *pmrA* mutations is that they prevent the Asp active site from being dephosphorylated by the phosphatase activity of PmrB.

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